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CALCIUM MOBILIZATION AND ENHANCED NATURAL KILLER FUNCTION IN LARGE GRANULAR LYMPHOCYTES RESULT FROM CROSS-LINKING the CD2 E-ROSETTE AND CD16 Fc-RECEPTOR

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difficult to firmly conclude whether these differences in signalling to resting T-cells via CD2 are due to the intrinsic properties of the antibody, or whether they could be due to the particular regions of the CD2 molecule recognized. For a series of reasons, and particularly from the investigations of a battery of CD2 of different isotypes, the second possibility appears to be much more likely, and thus different signals would be delivered to T-cells according to the conformational changes imposed on CD2. It is clear that distinct conformational changes, or reorientation of the molecule on the cell surface, can be imposed on CD2, according to the region on which antibodies bind, as revealed by events which can be termed epitope-specific modulation [3, 8]. These events could occur physiologically upon binding of CD2 to its natural ligand(s).

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T2.6 Calcium mobilization and enhanced natural killer function in large granular lymphocytes result from cross-linking the CD2 E-rosette and CD16 Fc-receptor

C.H. JUNE, J.A. LEDBETTER, P.S. RABINOVITCH, K.E. HELLSTROM, and I. HELLSTROM

The binding of monoclonal antibodies that recognize the CD2 E-rosette receptor results in T-cell proliferation, and in increased killing by cytotoxic T-lymphocytes and large granular lymphocytes (LGL) [1-4]. We recently found that in the case of LGL, a single antibody directed against 'internal' activation epitopes of the CD2 structure was sufficient to increase intracellular calcium concentration $[Ca^{2+}]_i$, whereas in the case of T-cells, combinations of CD2 antibodies against both 'internal' and 'external' epitopes were required [5]. The purpose of the present study was to evaluate the CD2 panel of antibodies to further define the mechanisms of LGL activation via the CD2 pathway. Our results indicate that only antibodies of the IgG3 isotype are capable of directly stimulating LGL calcium mobilization and natural killing, and furthermore, that this stimulation is dependent upon both the Fc and $F(ab')_2$ regions of the antibody, indicating a requirement for the simultaneous bridging of the CD2 E-rosette and CD16 FcR_{low} structures.

Using a sensitive assay to measure $[Ca^{2+}]_i$ in single cells [6], we previously found that stimulation of peripheral blood mononuclear cells (PBMC) with a combination of mitogenic CD2 antibodies resulted in a biphasic $[Ca^{2+}]_i$ response consisting of an early low-magnitude response in LGL, and a delayed high-magnitude response in T-cells [5]. We therefore tested each of the CD2 antibodies for effects on $[Ca^{2+}]_i$ (Fig. 1). The antibodies were tested at a saturating concentration, with or without additional cross-linking which was done in order to potentiate signal transduction. When tested singly, none of the antibodies was able to increase T-cell $[Ca^{2+}]_i$. In contrast, three types of LGL responses were observed (Fig. 1). The $[Ca^{2+}]_i$ of LGL was not altered in 14 cases (type 1 response), the $[Ca^{2+}]_i$ increased only after cross-linking in seven cases (type 2 response), and in two cases, a calcium response occurred which did not require cross-linking (type 3 response) (Table 1). We have previously demonstrated in this assay system that these alterations in LGL calcium metabolism occur in cells selected by positive or negative antibody selection [5]. The responses observed were distinct but the interpretations have some limitations. For example, antibody 449 391121 was of rat origin, and therefore could not be cross-linked with the second step reagent, and, furthermore, it is possible that some of the antibodies in the panel were of the lambda light chain, and similarly, would not have been

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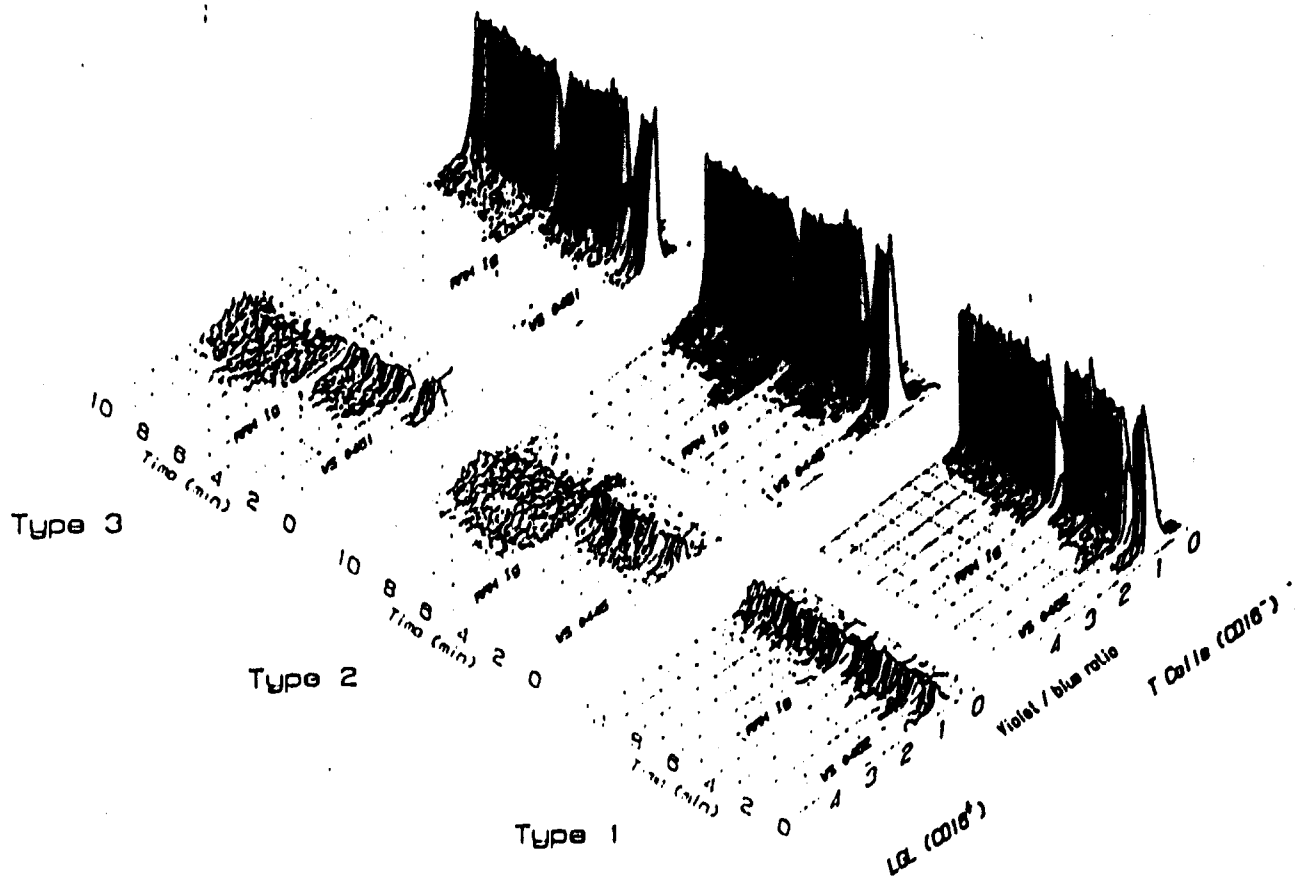

Fig. 1. Effects of CD2 antibodies on $[Ca^{2+}]_i$ in T-cells and LGL. Nylon wool purified T-cells were loaded with Indo-1 and labelled with phycoerythrin-conjugated, CD16 Fc-2 monoclonal antibody as described previously [5]. Results from real-time, dual-laser flow cytometric analysis are displayed as isometric plots consisting of 100 sequential histograms of cell number versus Indo-1 violet/blue fluorescence which is proportional to $[Ca^{2+}]_i$ [6]. LGL and T-lymphocytes were detected by gating on forward scatter and red (PE) fluorescence. At time = 1 min, Workshop antibodies (1:100) were added, followed by the addition of rat antimouse Ig kappa antibody 187.1 (60 μg/ml at t = 5 min). In this experiment 94 per cent of cells were T-lymphocytes and 6 per cent were LGL.

cross-linked. In addition, it is possible that some antibodies would have caused increased $[Ca^{2+}]_i$ in LGL at different doses or with more efficient cross-linking first- and second-step reagents.

The finding that both antibodies that caused a type 3 response were of the uncommon IgG3 isotype (Table 1) suggested that the Fc region of the antibody might be required for the response. An $F(ab')_2$ preparation of antibody 451 (9-1) was prepared [7]. This reagent retained specific and saturable binding to activated T-cells at ~1 μg/10^6 cells (not shown). The mitogenic combination of antibodies 9.6 and 9-1 were tested for their ability to affect $[Ca^{2+}]_i$ in LGL (Fig. 2). Intact antibody 9-1 alone was sufficient to cause a prompt increase in LGL $[Ca^{2+}]_i$, whereas the $F(ab')_2$ fragment did not alter $[Ca^{2+}]_i$ even at 50 μg/ml. The same result was obtained when intact antibody 9.6 was added simultaneously or sequentially to the $F(ab')_2$ preparation of antibody 9-1.

Previous studies have shown that mitogenic combinations of CD2 antibodies $T11_2$ and $T11_3$ are able to enhance killing of targets which are normally resistant to cytolysis by T-cell clones [2]. We tested the ability of individual CD2 antibodies to induce natural killing of the CD2-negative 2981 cell line derived from a lung adenocarcinoma [8] by freshly isolated PBMC (Table 1). Two antibodies, 451 and 456, mediated pronounced killing, and interestingly, both mediated type 3 LGL calcium responses. The killing associated with these antibodies was also observed with a separate CD2-negative target, the 2669 clone 13 melanoma line

Table 1. Functional effects of CD2 Workshop antibodies on LGL calcium mobilization and killing

Workshop antibody	Isotype	$[Ca^{2+}]_i$ response			NK killing
		Per cent Responding Cells	Maximum $[Ca^{2+}]_i$ (nM)	Type of response*	Per cent cytolysis
D66/1	G2b	72	391	2	0
OCH.217	M	—	—	1	4
9.6	G2a	—	—	1	6
35.1	G2a	—	—	1	8
39B21	G2a	—	—	1	0
9-2	M	—	—	1	2
9-1	G3	83	428	3	61
F92-3A11	G1	—	—	1	2
95-5-49	?	—	—	1	2
7A9	M	26	245	2	1
7E10	G2a	—	—	1	2
MOMO2A6 ($T11_3$)	G3	58	289	3	20
T11/3 PT2H9	G1	—	—	1	3
T11/3 T4-8B5	G2a	—	—	1	6
MT110	G1	76	368	2	6
MT26	G1	72	349	2	3
MT910	G1	70	360	2	0
NU-T1	?	78	321	2	1
TS1/8.1.1	G1	—	—	1	16
TS2/18.1.1	G1	—	—	1	9
CLB-T11/1	G1	—	—	1	2
SA1-11	G1	—	—	1	3
NU-TER	G1	42	297	2	4
— —	—	<5	130	—	8

*1. less than 5% 2. no change from baseline 3. medium only

[9], indicating that cytolysis could occur with multiple targets (not shown). In separate experiments, when tested on the same cell line at an E:T ratio of 10, antibody 9-1 still caused 50 to 78 per cent lysis (not shown). The mean ± S.D. cytolytic effect of PBMC from four different donors in the absence of antibodies was 13.3 ± 4.7 per cent. None of the CD2 Workshop antibodies caused >2 per cent lysis when tested in the absence of PBMC.

In further experiments, freshly isolated PBMC were separated into T-cells (>99 per cent $CD3^+$) and LGL (90 per cent $CD16^+$) by negative selection using a flow cytometer. The effector cells were added with antibody 9-1 (10 μg/ml) to the 2981 cell line at an E:T ratio of 10. The per cent lysis for T-cells, LGL, and T-cells plus LGL was 3, 52, and 45 per cent, respectively. The findings that T-cells did not mediate killing and were unable to augment LGL killing, indicate that the observed cytolytic activity is natural killing and is not due to cytotoxic T-lymphocytes.

The induction of NK cytolytic activity by antibody 451 (9-1) was further studied using the 2669 melanoma cell line as a target (Fig. 3). The effect of antibody 9-1 was blocked by 'external' epitope CD2 antibodies 9.6 and 35.1, and by CD16 antibody Fc-2 which binds to the low-affinity Fc receptor of LGL. Antibody MG21 mediates ADCC on this cell line [9], and has the same isotype as antibody 9-1. The ADCC mediated by MG21 was not blocked by CD2 antibodies but was blocked by CD16 Fc-2 (Fig. 3). Finally, the ability of PBMC 'armed' with the $F(ab')_2$ fragment of antibody 9-1 to mediate cytolysis on the 2981 cell line was compared with PBMC 'armed' with intact antibody. The per cent specific lysis for cells coated with: intact antibody 9-1 was 61 per cent; antibody 9-1 $F(ab')_2$, 2.6 per cent; 9-1 $F(ab')_2$ + MG21, 6.6 per cent; and antibody MG21, 2.2 per cent. The lysis by PBMC without added antibody was 10.4 per cent. Thus the $F(ab')_2$ fragment did not enhance cytolytic activity, and this could not be restored by antibody MG21, a non-

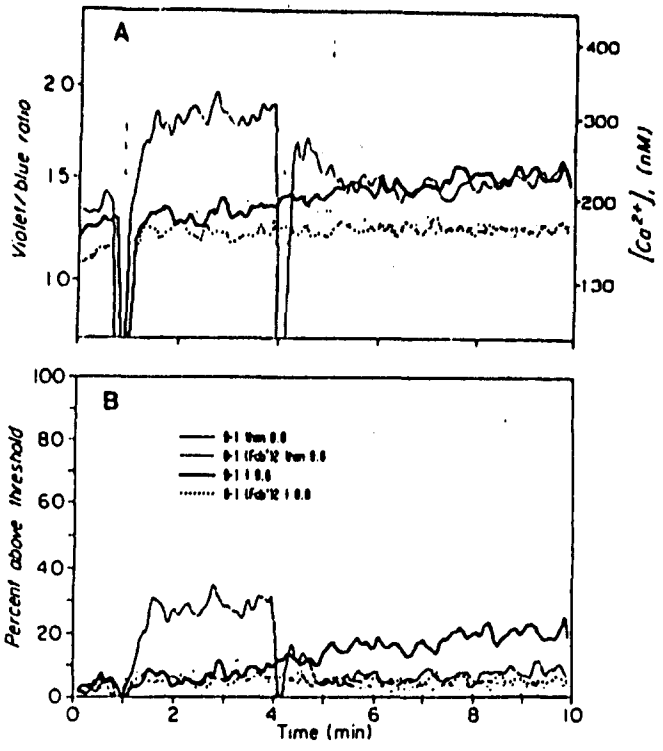

Fig. 2. Fc dependence of CD2-induced calcium mobilization in LGL. CD2 antibody 9-1 was added as the $F(ab')_2$ fragment (50 μg/ml) or intact (1 μg/ml) at t = 1 min with CD2 antibody 9.6 (10 μg/ml) added simultaneously at t = 1 min, or sequentially at t = 4 min. Nylon wool non-adherent cells were loaded with Indo-1, labelled with CD16 phycoerythrin-conjugated Fc-2, and analysed as in Fig. 1. The mean $[Ca^{2+}]_i$ and per cent responding cells for each experiment were calculated in panels A and B, respectively as described in [6].

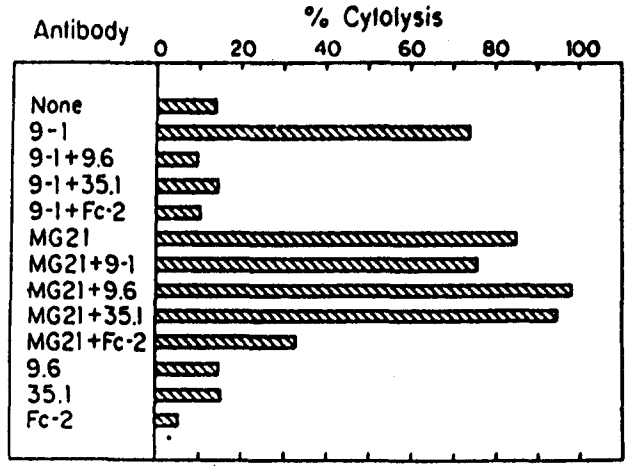

Fig. 3. Induction of enhanced killer function by CD2 antibodies. PBMC were used in a short-term Cr-release assay as effector cells at an E:T ratio of 100:1 [14,15]. The CD2-negative 2669 cl 13 melanoma cell line was labelled with ^{51}Cr and used as target cells. The assay was performed by adding successively target cells, antibody (10 μg/ml) and effector cells. After a 4-hour incubation, the mean per cent lysis of duplicate samples was determined by standard techniques. Antibodies tested were 9-1 (CD2, IgG3); 9.6 (CD2, IgG2a); 35.1 (CD2, IgG2a); Fc-2 (CD16, IgG2b) and MG21 (ganglioside Gs2, IgG3).

binding isotype matched antibody. The Fc region of M261 was demonstrated to bind to LGL and to mediate ADCC in Fig. 3. These results indicate that the cytolytic activity mediated by 9-1 clearly differs from ADCC, but like ADCC, appears to involve the CD16 FcR_{low} on LGL.

Taken together, our findings demonstrate that CD2 antibodies can activate the 'alternative' pathway on LGL by cross-linking the CD2 and CD16 surface structures. This activation was manifest biochemically by increased $[Ca^{2+}]_i$ and biologically by activation of the lytic programme of LGL. There is precedent for Fc receptor-mediated modification of activation signals where, for example, the cross-linking of the B-cell antigen receptor with the Fc receptor has been shown to alter the $[Ca^{2+}]_i$ response to stimulation by anti-immunoglobulin [10]. The question remains as to whether the activation signal for LGL is transmitted through the CD2 structure, the Fc structure, or through a third party structure equivalent to the CD3 structure of T-cells. On T-cells, it appears that the CD2 molecule requires a CD3-associated structure in order to transmit a signal [11]. There is precedent for signal transduction through Fc receptors in that Fc receptor antibodies have been shown to cause membrane depolarization and increased $[Ca^{2+}]_i$ in a murine macrophage line [12, 13]. Finally, the present study was unable to resolve whether a particular epitope of the CD2 molecule is requisite to achieve LGL activation, or whether the bridging of any CD2 epitope to the CD16 structure will result in activation.

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T2.7 Enhancement of cytolytic activity by bridging the CD2 sheep erythrocyte binding protein and the CD16 Fc receptor

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Human T-cells can be activated through a pathway involving the CD2 sheep erythrocyte receptor [1]. Evidence for this has come from observations that certain combinations of CD2 antibodies can deliver a signal that rapidly increases intracellular free ionized calcium concentration $[Ca^{2+}]_i$ [2, 3]. We have observed previously that binding of two antibodies specific for distinct CD2 epitopes is required in order to activate resting $CD3^+$ mature T-cells, whereas binding of the single CD2 antibody 9-1 is sufficient to activate resting $CD3^-$ large granular lymphocytes (LGL) [3]. We now provide evidence that the IgG3 antibody 9-1 binds simultaneously to CD2 and to the CD16 Fc receptor co-expressed on LGL. Cross-linking and co-modulation of CD2 and CD16 induces increased $[Ca^{2+}]_i$ and activation of the lytic programme.

We tested the ability of CD2 antibodies to induce increased $[Ca^{2+}]_i$ in cultured IL-2 dependent $CD3^+/CD16^-$ MHC-non restricted cytolytic T-cell clones and $CD3^-/CD16^+$ LGL clones. Both CD2 antibodies 9.6 and 9-1 were required in order to induce increased $[Ca^{2+}]_i$ in $CD3^+/CD16^-$ cells, whereas binding of the single antibody 9-1 was sufficient to trigger a response in $CD3^-/CD16^+$ cells (Table 1). These results were consistent with responses observed previously in the corresponding populations of resting peripheral blood lymphocytes [3]. The cultured $CD3^-/CD16^+$ LGL did not respond after stimulation with the IgG2a CD2 antibody 9.6 or with the IgG3 HLA class-I antibody 60.5 (Table 1). Pre-incubation of $CD3^-/CD16^+$ cells with the CD16 Fc receptor antibody FC-2 consistently inhibited the 9-1 induced $[Ca^{2+}]_i$ response by 70 per cent, suggesting that an interaction with Fc receptors was required for activation.

The dual binding of antibody 9-1 to CD2 and CD16 was demonstrated in cross-blocking and co-modulation experiments. Pre-incubation of $CD^{3-}/CD16^+$ cells with antibody 9-1 for 30 min at 4°C did not inhibit the binding of the CD16 antibody Leu-11a. However, incubation with antibody 9-1 for one hour at 37°C was followed by nearly complete disappearance of Fc receptors as detected by antibody Leu-11a (Fig. 1(A)). Modulation of CD16 Fc receptors with antibody FC-2 did not affect the expression of CD2 as detected by antibody 9-1. Thus there is no innate association between CD2 and CD16. It was of interest that co-modulation of CD16 was also induced by the IgG3 HLA class I antibody 60.5.

Given that cross-linking of CD2 and CD16 delivers an activation signal to LGL, it was of interest to examine the functional effects of antibody 9-1 in cytotoxicity assays. The K562, HEL, CHAN, and NALM-1 cell lines were selected as targets because they do not express CD2. Spontaneous lysis of both NK-sensitive and NK-resistant targets mediated by peripheral blood lymphocytes was